Docket No. 46745 (1758)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: J. Weidanz, et al.

SERIAL NO.: 08/813,781 EXAMINER: M. Lubet

FILED: March 7, 1997 GROUP: 1644

FOR: FUSION PROTEINS COMPRISING BACTERIOPHAGE COAT PROTEIN AND A SINGLE-CHAIN T CELL RECEPTOR

THE HONORABLE COMMISSIONER OF PATENTS AND TRADEMARKS
WASHINGTON, DC 20231

SIR:

DECLARATION PURSUANT TO 37 CFR 1.131

The undersigned declare as follows:

1. We are co-inventors of the above-identified application (hereafter the "subject application"). Jon A. Weidanz and Hing Wong are Senior Scientists with Sunol Molecular Corporation, Miramar Florida. Hing Wong is currently President and CEO of that corporation.

2. As we understand it, the subject application discloses and claims, among other things, fusion proteins in which bacteriophage coat protein is covalently linked (ie. fused) to a soluble single-chain T-cell receptor. As one example, the subject application discloses and claims, a single-chain T-cell receptor linked to a bacteriophage VIII coat protein ("single chain fusion protein").

3. We have reviewed the Patent Office Action ("Office Action") dated June 23, 1999 issued in connection with the subject application. As we understand the Office Action, the patent Examiner rejected claims 1, 2, 4, 7-8, 14, 18, 19, 20, 61 and 67 as obvious in view of WO 96/18105 ("Strominger") taken with other references

cited in the Office Action. We understand that Strominger is a PCT application having an issue date of 13 June 1996.

4. We have read Strominger. As we understand it, Strominger discloses a specific single-chain T-cell receptor that binds to an MHC peptide ligand.

5. The invention described and claimed in the subject application was conceived and reduced to practice in the United States prior to June 1996.

6. For example, well before June 1996, we recognized that it was possible to make a recombinant T-cell receptor (TCR) protein in which a V-alpha chain was fused to a V-beta C-beta chain by a peptide linker sequence. It was also recognized before the June 1996 date that a bacteriophage coat protein could be fused to the V-beta C-beta chain to produce the single-chain fusion protein. As understood, use of the bacteriophage gene VIII coat protein could substantially improve features of the fusion protein. As disclosed throughout the subject application, that recognition helped us make and use the single-chain fusion protein.

7. The claimed single-chain fusion protein was made in the United States well before Strominger's June 1996 publication date.

8. For example, conception of the single-chain fusion protein was accomplished well before Strominger's publication date. Attached as **Exhibit 1** is a true and accurate copy, with dates deleted, of notes made by one of us before June 1996. For example, see pages 1 and 2 of the notes in which is shown, among other things, drawings of single-chain fusion protein. Also indicated in the notes, among other things, are steps for making vector encoding peptide linker sequence fused to the bacteriophage gene VIII protein. The vector is referred to as pKC27. Construction and use of the pKC27 vector is further exemplified by the subject application. For

instance, see Example 2 and particularly part E which discloses work performed well before the June 1996 date. See also Figure 2 of the application (outlining production of pKC27 and other vectors).

9. One of us prepared sequence encoding TCR V-alpha chain well before Strominger's June 1996 publication date. Attached as **Exhibit 2** is a true and accurate copy of notes, with dates deleted, prepared by one of us well before that date. The notes show, among other things, successful production of sequence encoding the V-alpha chain. These manipulations were performed well before the June 1996 date and are described throughout the subject application, for instance, see Example 1.

10. Well before the June 1996 publication date of Strominger, one of us fused sequence encoding the V-alpha chain into the pKC27 vector. That manipulation led to production of pKC42 vector. The pKC42 vector particularly encodes sequence in which the V-alpha chain is fused to the peptide linker. That linker is fused to the bacteriophage gene VIII protein. Attached as **Exhibit 3** is a true and accurate copy of notes made by one of us, with dates deleted, describing steps taken to make the pKC42 vector among other things. These steps were undertaken well before the June 1996 date and are further exemplified in the subject application. For instance, see Example 2 and particularly section E. See also Figure 2 of the subject application (outlining production of pKC42 from the pKC27 vector).

11. We made vectors that encode the TCR V-beta C-beta chain well before Strominger's June 1996 publication date. Attached as **Exhibit 4** is a true and accurate copy of notes made by one of us, with dates deleted, that shows, among other things, steps taken to make pKC30 vector. These manipulations were conducted well before the June 1996 date and are further exemplified in the subject application. See Figure 2, for example.

12. In experiments performed well before Strominger's June 1996 publication date, we made vectors that encode the V-beta C-beta chain fused to the bacteriophage gene VIII protein. Attached as **Exhibit 5** is a true and accurate copy of a note made by one of us, with dates deleted, which shows, among other things, manipulation of a vector called pKC34.3. The pKC34.3 vector is a specific isolate of pKC34 and it is further exemplified in Figure 2 of the subject application (see the step for making pKC44 from the pKC42). The note particularly describes treatment of pKC34.3 with restriction endonucleases to isolate a fragment encoding the V-beta C-beta chain fusion protein. Steps taken to make the pKC34.3 vector were performed well before the June 1996 date.

13. Well before Strominger's June 1996 publication date, we inserted sequence encoding the V-beta C-beta bacteriophage gene VIII fusion protein into the pKC42 vector. Attached as **Exhibit 6** is a true and accurate copy of notes made by one of us, with dates deleted, showing, among other things, production of the pKC44 vector (encodes single-chain fusion protein). In particular, pages 1-5 of the notes show manipulation of specific pKC42 vectors (42.1, 42.2, and 42.3) and use of those vectors as recipients of sequence encoding the V-beta C-beta bacteriophage gene VIII construct. These results are further exemplified by the subject application. For instance, see Example 2 and particularly section E. See also Figure 2 of the application (showing production of pKC44 from pKC42).

14. We made the single-chain fusion protein in the United States well before the June 1996 publication date of Strominger.

15. For example, attached as **Exhibit 7** is a true and accurate copy of notes made by one of us, with dates deleted, that shows, among other things, manufacture of the single-chain fusion protein well before Strominger's June 1996 date. In particular, pages 1-4 of the notes show expression of the single-chain fusion protein

encoded by pKC44 as evidenced by a Western Blot. Pages 5 and 6 of the notes show purification of that protein using an immuno-affinity column. These manipulations are further exemplified by the subject application. For instance, see Examples 4, 5, 6 and Figures 7-13 which disclose work performed well before June 1996.

16. We hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 12/14/99 — Hing C. Wong

Date: 12/1/99 — Jon A. Weidanz

Date: 12/14/99 — Kimberlyn Card

#130721

pKC15.1 with SpeI and XhoI

Objective: To begin work on a single chain construct [diagram] L=linker S=gene of interest
as an alternative to the current Fab constructs:
pKC30.1 [diagram] pKC15.1 [diagram]
The fusion protein may not be made efficiently as the 2nd protein after a single promoter. A single chain construct may overcome this type of problem. The two proteins will be seen as a single protein connected together by a linker sequence.
Step 1: Cut vector with gene VIII to allow linkers as insert.

Method/Materials: pKC15.1 DNA prep @ 390 ng/µl
SpeI @ 15 U/µl
XhoI @ 20 U/µl
BSA (1:10)
B+H Buffer 2
purified water

Decided to cut 10 µg of pKC15.1 for digest:

25.3 µl pKC15.1 (10 µg)
3.3 µl SpeI
2.5 µl XhoI
12 µl BSA
12 µl buffer 2
64 µl water
120 µl total

Incubated in 37°C waterbath
2:50 - 4:50 heated inactivated
@ 65°C for 20 minutes

Conclusion: pKC15.1 cut with SpeI and XhoI.

KC

To Page No. N/A

Witnessed & Understood by me,	Date	Invented by	Date
		Recorded by	

Project No. RE[redacted]
Book No. D-80[redacted] TITLE PCR amplification [redacted] and β chains for single chain construct

2

From Page No. N/A

Objective: To amplify out the DO11.10 cell line's TCR chains by PCR. The α chain will contain only the variable region with SfiI and SpeI restriction sites for cloning. The β chain will contain the variable region and most of the constant region (ending just before the final cysteine) with XhoI and XmaI restriction sites for cloning.

(margin: for single chain)

Materials/Method: PCR buffer II
MgCl$_2$ sol'n
10mM dNTPs
pKC16.7 (1:100)
pKC18.7 (1:100)
purified water

KC114 (α 5' front w/ SfiI)
JWTCRα23 (α back w/ SpeI)
KC115 (β front with XhoI)
KC118 (β back with XmaI)
@ 10 pm/μl

Set up 10 reactions for each. Made 10x pool and then added taq. Tubes put in thermocycler once @ 96°C

α	β
100 μl buffer	"
80 μl MgCl$_2$	"
20 μl dNTPs	"
10 μl KC114	10 μl KC115
10 μl JWTCRα23	10 μl KC118
20 μl 16.7	30 μl 18.7
755 μl water	745 μl water
+ taq (0.5 μl/tube)	"

attached: gel picture

lane 1 = λBstEII
2 = α PCR (1/10 1 rxn)
3 = β PCR (1/10 1 rxn)

Conclusion: PCRs worked!

[redacted]

RE701-0-801-62 KC

1 min.
96°
1 min.
60°
20 °C
1 min. 72°
10 min. 72°
x 15 cycles
x 1 cycle

KC

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Witnessed & Understood by me, | Date | Invented by | Date
Recorded by

BEST AVAILABLE COPY

[redacted]DNA

Objective: To pool and digest the 2 and 3 PCR products to prepare for ligation.

Method/Materials: α PCR's 0-801-62
β PCR's 0-801-62
SfiI @ 20U/μl
SpeI @ 15U/μl
XmaI @ 10U/μl
XhoI @ 20U/μl
BSA (1:10)
Buffer 1
Buffer 2
purified water

Method:

α digest
5 μl SfiI
7 μl SpeI
24 μl BSA
24 μl buffer 2
135 μl water
45 μl [redacted]C DNA
240 μl

↓

2 hours @ 37°C
2 hours @ 50°C

β digest
10 μl XmaI
5 μl XhoI
30 μl BSA
30 μl buffer 1
150 μl water
45 μl DNA
300 μl

↓

4 hours @ 37°C

Heat inactivated @ 65°C for 20 minutes.

Conclusion: Digests performed as described.

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Witnessed & Understood by me,	Date	Invented by	Date
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Non/A

Objective: To anneal a pair of oligos together to make the linker sequence to the single chain construct.

Materials/Method: JA301 @ 10pm/ul
JA302 @ 10pm/ul
2x annealing buffer

Mixed: 25ul buffer
12.5ul JA301
12.5ul JA302
50ul total

added to thermocycler once @ 96°C

3 min. 10 min.
96°C
30 min.
2 min.
30°C

Attached: Gel picture.
(3% nusieve/1% seachem)
Want to see two oligos annealed

RE701-0-801-70

lane 1 = MspT
2 = JA301
3 = JA302
4 = annealed oligos

Conclusion: There appears to be annealed oligos present. The oligo insert is @ 25pM/ul.

☆ Want to convert oligo concentration from pM/ul to ng/ul.

165 bp × 1660 = 42,900 g/mole
= 3 × 10⁴ pg/pM

$$\frac{4.3 \times 10^4 \text{ pg}}{\text{pM}} \times \frac{25 \text{ pM}}{\text{ul}} = 1.08 \times 10^5 \text{ pg/ul}$$

∴ 108 ng/ul

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Objective: To ligate the linker sequence into pUC19.1 x SpeI x XhoI to form the base of a single chain construct.

Method/Materials: pUC19.1 x SpeI x XhoI D-801-67
annealed linker oligos D-801-70
T4 ligase
T4 ligase buffer
purified water

Set up 4 ligations. Remember oligo is not phosphorylated; vector is ∴ use excess insert.

Insert @ ~100ng/µl ↙ 165bp — dilute 1:10 to use

Vector @ [redacted] 50ng/µl — 3310 bp

∴ use 50ng vector: 1 ng insert = 1:1 ratio

	1:0	1:3	1:10	1:30
vector	3µl (150ng)	3µl (150ng)	3µl (150ng)	3µl (150ng)
insert	—	0.33µl (3ng)	3µl (30ng)	0.9µl (∴ insert = 90ng)
ligase	1µl	1µl	1µl	1µl
buffer	1µl	1µl	1µl	1µl
water	5µl	4.67µl	2µl	4.1µl
	10µl	10µl	10µl	10µl

Mixed all components except ligase. Heated @ 65°C for 5 minutes and put on ice. Added ligase. Incubated in 16°C water bath overnight

Conclusion: ligation set up as described

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Objective: To transform XL1B cells with the ligated DNA from D-801-71

Material/materials: XL1B competent cells
ligated DNA D-801-71
B1-1 media
B1-1 + amp plates

Same method as on D-801-50.

Conclusion: Colonies counted.

1:0	10 µl = 48		1:10	10 µl = 116
	100 µl = 1023			100 µl = 999
1:3	10 µl = 38		1:30	10 µl = 9
	100 µl = 429			100 µl = 74

The numbers trend (high colony # → low colony # as insert concentration rises) look promising.

Selected 10[cc] 12 colonies from 100 µl 1:30 plate and grew 5ml overnight cultures with amp + tet. for each.

KC

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Witnessed & Understood by me,	Date	Invented by	Date
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Title: Pool and Digestion of [redacted] (114/119)

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Objective: To pool and digest the Vκ PCR products to prepare for ligation.

Method/Materials: Vκ PCR products D-816-15
SfiI @ 20u/ul
SpeI @ 15u/ul
Buffer 2
BSA (1:10)
purified water
Qiaspin PCR Purification kit

① PCR products pooled and cleaned up using kit. Volume = 50 ul

② κ digest
7ul SfiI
7ul SpeI
24ul BSA
24ul buffer 2
35ul water
45 ul DNA
240ul

Cut for 2 hours @ 37°C.
Cut for 3 hours @ 50°C.

③ Heat inactivated for 20 minutes @ 65°C.

Conclusion: Digestion performed as described.

12C

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Witnessed & Understood by me,	Date	Invented by	Date
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N/A

Objective: To gel purify and then quantitate the cut V$_\kappa$ PCR products.

Method/Materials: V$_\kappa$ (1114/1119) x Spe I x Sal I D-816-17
1% prep agarose gel
Qiaquick Spin Gel Purification kit Qiagen

① DNA was precipitated and resuspended in 30 µl 1/10 TE buffer. Ran prep gel with λBst E II digest. Cut out fragment of interest.

② DNA purified according to manufacturer's instructions.

③ Ran 1% gel to quantitate DNA.

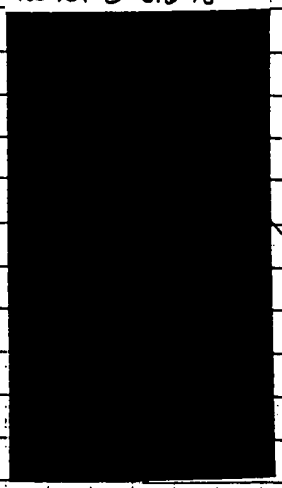
Gel: 1 = λBst E II (2 x 2 µg)
2 = " (1 µg)
3 = " (0.5 µg)
4 = " (0.25 µg)
5 = DNA (5 µl)

Attached: Gel pictures.

Conclusion: The 5 µl of sample DNA looks like the 2 µg line of the marker so 5 µl = 28 ng.
The DNA is @ 5.5 ng/µl.

Lanes A+B are PCR products for 1114/1116 and 1118/1118. See D-816-19-20 for PCR reactions.

RE701-D-816-18



RE701-D-816-18

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Witnessed & Understood by me,	Date	Invented by	Date
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Gel Purification - Digestion of 114/126x

Page No: N/A

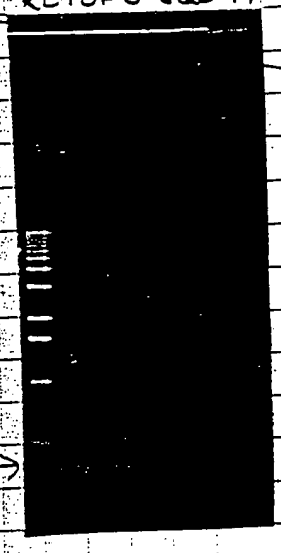
Objective: to gel purify and quantitate 114/126 cut with SfiI and SpeI

Method/Materials: 114/126x SfiI x SpeI D-825-71D
Qiaquick Spin Purification kit - Qiagen

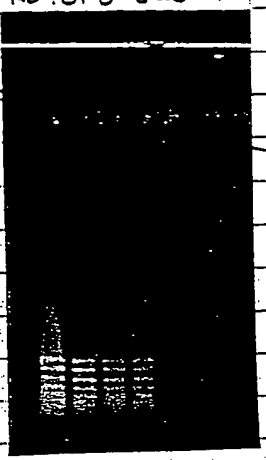
1% Rao DNA in 2% prep agarose gel Isolated fragment of interest
Purified DNA using kit.

Attached: Gel pictures.

RE701-D-825-71



RE701-D-825-71



For gel B, lane 1 = MspI (4ug)
2 = " (2ug)
3 = " (1ug)
4 = " (0.5ug)
5 = 114/126 (2ul)
6 = " (5ul)

Conclusion: 5ul looks like 1/3 of the 3rd band down in the 0.5ug mwm lane.

∴ 404 = 92.6ng/ug mwm
4361 ÷ 2 ÷ 3 = 15.4ng/5ul sample
114/126 @ = 3ng/ul

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Witnessed & Understood by me,	Date	Invented by	Date
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Objective: To ligate 114/126(V2) into pKC27.8

Materials: 114/126 x Sfi I x Spe I D-825-70 @ 3ng/µl
pKC27.8 x Sfi I x Spe I D-816-26 @ 35ng/µl
T4 ligase
T4 ligase buffer

Method: Set up 4 ligations

Vecto ≅ 3500 bp. Insert ≅ 350 bp. Ratio = 1:10 (vecto:insert)
Same method as on D-825-35 except incubated 1 hour @ room temperature.

	1:0	1:1	1:3	3:1
vecto	4.4	4.4	4.4	13.1
insert	—	4.1 —	14.0	4.1
buffer	1	2	2.2	2.2
ligase	1	1	1	1
water	3.6	7.9	0.4	1
	10µl	20µl	22µl	22µl

Conclusion: Ligations set up as described.

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Witnessed & Understood by me,	Date	Invented by	Date
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Objective: To transform XL1B cells with pKC42 ligated DNA.

Materials: pKC42 ligation D-825-#2
XL1B cells
BL-1 media

Method: Same as D-825-28, #① and ②

Conclusion: Colonies counted

1:0	10 µl = 6	1:3	10 µl = 388*
	100 µl = 58		100 µl = TNTC
1:1	10 µl = 243	3:1	10 µl = 181
	100 µl = TNTC		100 µl = TNTC

Conclusion: Transformation number is look very good.
Grew 10 colonies on * plate

KC

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Witnessed & Understood by me,	Date	Invented by	Date
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Objective: To isolate plasmid DNA and perform analytical digestion on pKC+2 candidates.

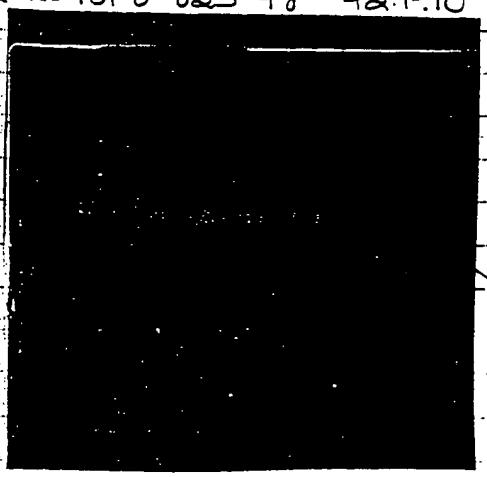
Materials/Method: Used Qiagen's Qiaprep Plasmid Extraction kit to isolate plasmid DNA from cell pellets of candidates.
Made 12x pool of reagents:

3 µl SfiI (@20U/µl)
3.6 µl SpeI (@13U/µl)
18 µl Buffer 2
18 µl BSA (1:10)
17.4 µl water
→ 5 µl to each + 10 µl sample DNA (0.2 µl vector DNA)

Incubated @ 37°C for 1 hour. Incubated @ 50°C for 1 hour

Attached: Gel picture.

RE701-0-825-78 42.1-10



Lane 1 = MspI (1 µg)
2-11 = candidates #1-10
12 = vector only (2-7-8)

Conclusion: 10/10 candidates have an insert of the correct size.
Selected #1, 2, and 3 to focus on. Streaked out for single colonies.
Will prepare DNA for sequencing.

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Objective: To pool and digest the Vβ Cβ PCR products for ligation.

Method/Materials: Vβ PCR products (122/116) D-8/e-20
" (122/118) "
XmaI @ 10U/ul
XbaI @ 20U/ul
Buffer 1
BSA (1:10)
purified water
QiaspinPCR Purification kit Qiagen

① PCR products pooled and cleaned up using kit. Volume = 50 ul

② Digest (set up for 122/116 and 122/118 separately)

10 ul XmaI
5 ul XbaI
30 ul BSA
30 ul buffer 1
180 ul water
45 ul DNA
300 ul

Cut for 4 hours @ 37°C.

③ Heat inactivated 20 minutes @ 65°C.

Conclusion: Digestions performed as described.

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Witnessed & Understood by me,	Date	Invented by	Date
		Recorded by	

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Objective: To gel purify and then quantitate the cut Vβ PCR products.

Materials: Vβ (122/116) x XhoI x XmaI O-816-24
Vβ (122/118) x XhoI x XmaI O-816-24
Same as on O-816-18.

Method: Same as on O-816-18.

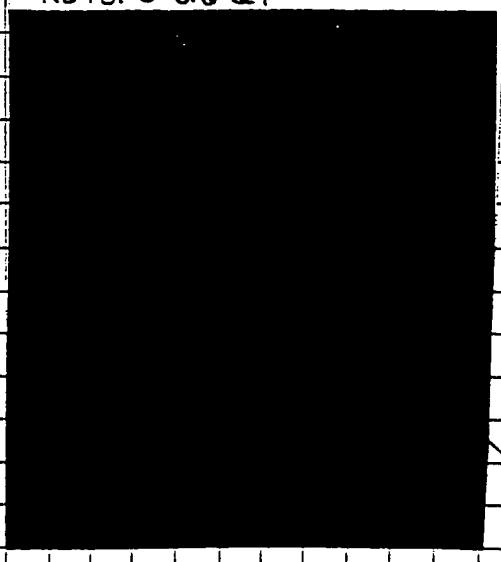
Gel: 1 = λBstEII (2 μg)
2 = " (1 μg)
3 = " (0.5 μg)
4 = " (0.25 μg)
5 = 122/116 (5 μl)
6 = 122/118 (5 μl)

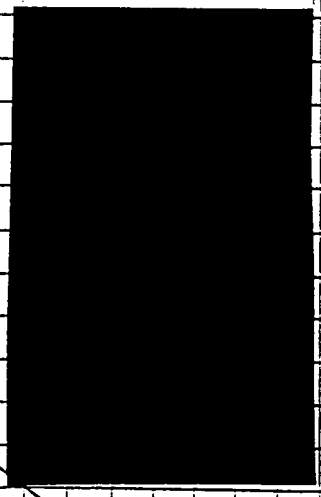
Conclusion:

122/116 looks like the 2 μg band
x 3 = 28 ng x 3 = 84 ng.
∴ 1 μl = 16.8 ng.

122/118 looks like the 2 μg band
= 28 ng.
∴ 1 μl = 5.6 ng.

Attached: Gel pictures





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Witnessed & Understood by me,	Date	Invented by	Date
		Recorded by	

Page No. N/A

Objective: To ligate V_β(122/116) with pKC24.2. V_β(122/116) = β chain w/ sig.

Materials/ method: V_β(122/116) x Xho I x Xma I D-816-29 @ 16.8 ng/µl
pKC24.2 x Xho I x Xma I @ 41.2 ng/µl
T4 ligase
T4 ligase buffer
purified water

① Same as step ① on D-816-27

② Set up 4 ligations. Vector ≅ 3900. Insert ≅ 750 bp. Ratio = 5.5

	1:0	1:1	1:3	3:1
vector	3.4µl (140ng)	2.4	3.4	10.2
insert	1	1.5µl (255ng)	4.5	15
buffer	1µl	1	1	2
ligase	1µl	1	1	1
water	4.6µl	3.1	0.1	5.3
	10µl	10µl	10µl	20µl

Conclusion: Ligations set up as described.

ICC

To Page No. N/A

Witnessed & Understood by me,	Date	Invented by	Date
		Recorded by	

Objective: To ligate Vβ(122/118) with pKC24.2. Vβ(122/118) = β chain variable region.

Materials/Method: Vβ(122/118) x NcoI x XmaI 0-816-29 @ 5.6ng/µl
pKC24.2 x NcoI x XmaI @ 41.2ng/µl
T4 ligase
T4 ligase buffer
purified water

① Same as step ① on 0-816-27

② Set up 3 ligations (1:0 was already set up on 0-816-30).
Vector ≅ 3900bp. Insert ≅ 720bp. Ratio = 5.5

	1:1	1:3	3:1
vector	3.4 µl (140ng)	3.4	10.2
insert	~~1~~ RC 4.6 µl	13.8	4.6
buffer	1 µl	2	2
ligase	1 µl	1	1
water	—	—	2.2
	10 µl	20.2 µl	20 µl

Conclusion: Ligations set up as described.

RC

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Witnessed & Understood by me, | Date | Invented by | Date
Recorded by 0-816

Objective: To transform χ1776 B cells with the ligated DNA from D-816-30 and D-816-31

Materials: ligated DNA from D-816-30 (pKC29)
ligated DNA from D-816-31 (pKC30)
χ1776 cells
B17-1 media
B12-1 + amp. plates

Method: Same as on D-816-28.

Conclusion: Colonies counted next day.
Picked 10 colonies from each ligation to grow D.N.

1:0 100 µl = 0
100 µl = 9

pKC29

1:1 10 µl = 6
100 µl = 97

1:3 10 µl = 2
100 µl = 102

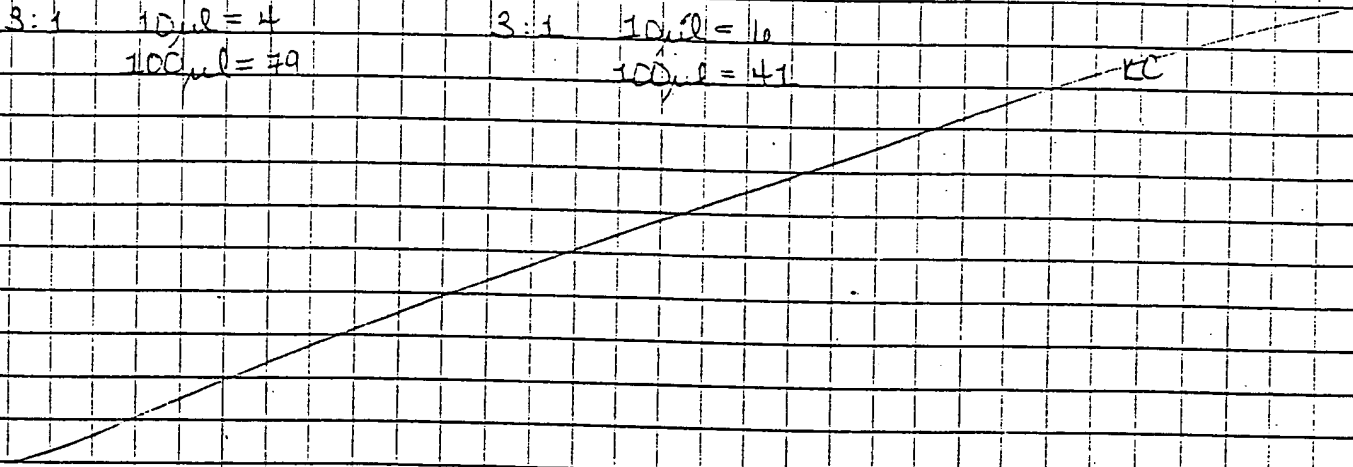
3:1 10 µl = 4
100 µl = 79

pKC30

1:1 10 µl = 2
100 µl = 316

1:3 10 µl = 0
100 µl = 42

3:1 10 µl = 16
100 µl = 41



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Project No. RE701 TITLE Miniprep Digestion of pKC29 + pKC30
Book No. 0-816

Page No. N/A

Objective: To screen pKC29 and pKC30 candidates for insert

Method/materials: ① Used Qiaspin Plasmid Extraction Kit from Qiagen to isolate plasmid DNA

② Cut 1/10 of DNA with XhoI and XmaI to look for an insert of ≈ 700 bp.
Made 24 pool:

6 μl XhoI
12 μl XmaI
12 μl BSA (1:10)
12 μl Buffer 1
75 μl purified water
120 μl

↓
5 μl to each digest + 10 μl DNA
+ 3 μl control
(control = pKC27.2)

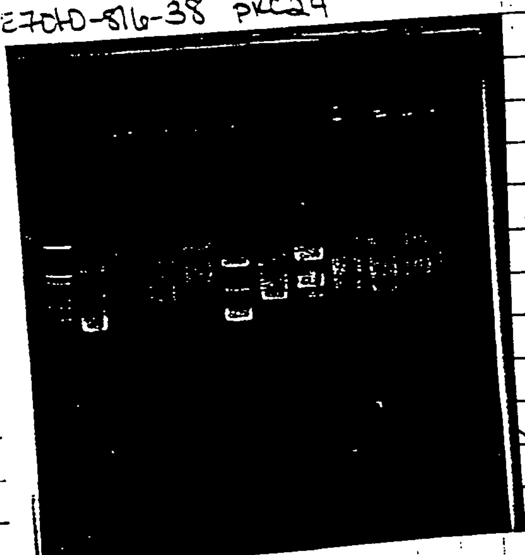
③ Incubated @ 37°C for 2 hours
④ Ran 1% agarose gel; lane 12 = control

Conclusion: For 29 (gel on left), 6/10 candidates have an insert of the correct size. Selected candidates # 2, 4, 8 and streaked out single colonies.

For 30 (gel on right), 6/10 candidates have an insert but candidate #1's insert is larger than the others. Selected candidates # 2, 3, 6 and streaked out single colonies.

Attached: Gel picture

RE701-0-816-38 pKC29



Attached: Gel picture

RE701-0-816-38 pKC30

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Project No. [redacted]
Book No. D-825 TITLE Digestion of pKC343 and pKC352 with XhoI and ECoRI

Page No. N/A

Objective: To digest pKC343 and pKC352 with XhoI and ECoRI

Method/Materials: Decided to cut 20µg of DNA each. Set up digests:

100µl pKC343 (≈18µg total) D-825-67	45µl pKC352 (20µg) D-825-67
5µl XhoI (@ 20U/µl)	5µl XhoI
1µl ECoRI (@ 100U/µl)	1µl ECoRI
14µl Buffer 2	14µl Buffer 2
14µl BSA (1:10)	14µl BSA (1:10)
16µl water	101µl water
140µl total	140µl total

Incubated @ 37°C from 2-4pm.
Heat inactivated @ 65°C for 20 minutes.
Precipitated DNA and resuspended in 50µl 1/10 TE buffer.

Conclusion: DNA digested as described

KC

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Plasmid Prep. of pKC4[redacted], 2 and 3

Page No N/A

Objective: To isolate plasmid DNA from pKC45 1, 2 and 3

Method/Materials: Same as on D-825-45

Conclusion: OD scans were not printed out

pKC42.1 (1:100) 260 = 0.2053 260/280 = 1.59
280 = 0.1288

0.2053 × 50 × 100 = 1026.5 ng/µl × 200 µl = 205.3 µg

pKC42.2 (1:100) 260 = 0.1896 260/280 = 1.78
280 = 0.1063

0.1896 × 50 × 100 = 948 ng/µl × 200 µl = 189.6 µg

pKC42.3 (1:100) 260 = 0.1658 260/280 = 1.81
280 = 0.0915

0.1658 × 50 × 100 = 829 ng/µl × 200 µl = 165.8 µg

Conclusion: DNA isolated as described

KC

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...: To digest the pKC42 candidates #1, 2, 3 with XhoI and EcoRI

...d/Materials: Decided to cut 20µg of each.
Set up digests.

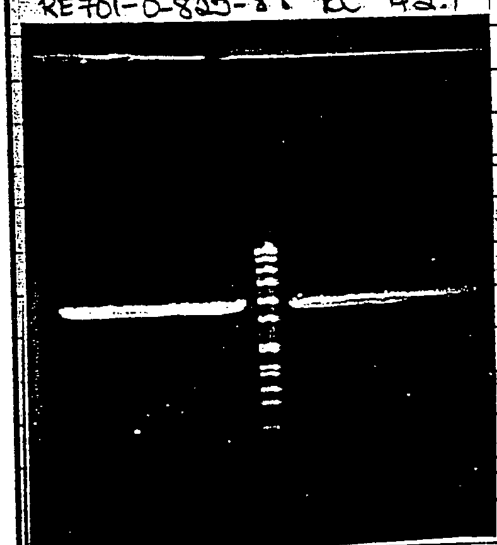
10 µl pKC42 1	21.1 µl pKC42 2	24.1 µl pKC42 3
5 µl XhoI (@ 20U/µl)	5 µl XhoI	5 µl XhoI
1 µl EcoRI (@ 100U/µl)	1 µl EcoRI	1 µl EcoRI
12 µl Buffer 2	12 µl Buffer 2	12 µl Buffer 2
12 µl BSA (1:10)	12 µl BSA (1:10)	12 µl BSA (1:10)
70.5 µl water	68.9 µl water	65.9 µl water
120 µl total	120 µl total	120 µl total

Incubated @ 37°C from 10:40 am to 1:40 am.
Heat inactivated @ 65°C for 20 minutes
Precipitated DNA and resuspended in 50 µl 1/10 TE buffer.

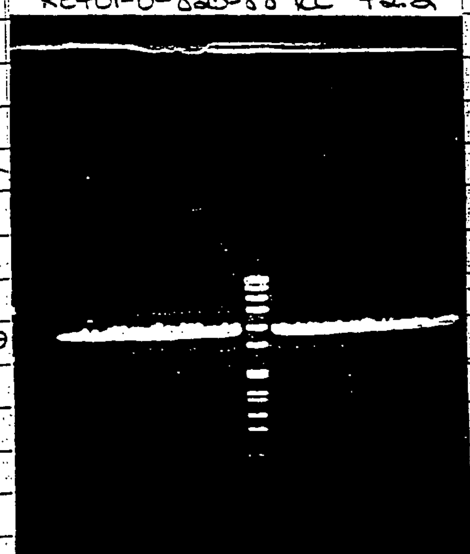
Ran DNA in 1% prep agarose gels. Cut out fragments of interest and froze

Attached: Gel pictures. Conclusion: DNA digested as described.

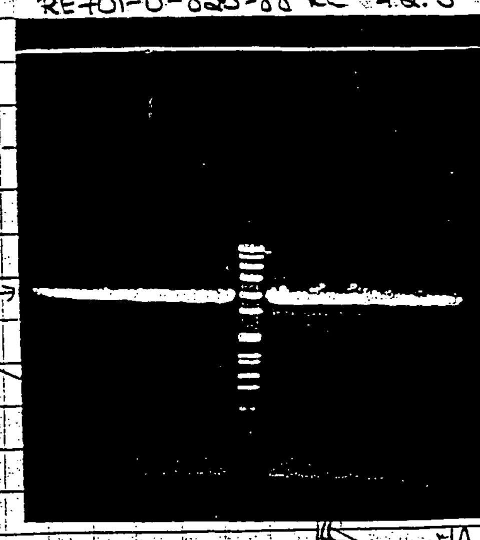
RE701-0-825-88 KC 42.1



RE701-0-825-88 KC 42.2



RE701-0-825-88 KC 42.3



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Gel Purification + Dephosphorylation of pKC42 1, 2, 3

Page No. N/A

Objective: To gel purify and then dephosphorylate pKC42 1, 2, 3 digested with XhoI and EcoRI.

Method/Materials: pKC42 1 x XhoI x EcoRI O-825-88
pKC42 2 " "
pKC42 3 " "
Qiaquick Spin Gel Purification Extraction Kit - Qiagen

① Purified DNA out of isolated gel fragments on O-825-88 using kit. Final volume = 100 µl each.

② Dephosphorylated each:

100 µl DNA
1 µl CIAP
12 µl Buffer 12
7 µl water
120 µl total

Incubated @ 37°C from 12:25pm to 2:05pm.
Cleaned up using Qiaquick Spin PCR Purification kit.
Final volume = 100 µl each.

Will quantitate cut DNA after the correct clone has been identified by sequencing. [redacted] see O-825-94 J.K. Card

Conclusion: DNA was gel purified and dephosphorylated.

KC

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* For previous work on this project, please see DE701-D-825

Objective: To ligate β-gene VIII into pKC42.3 to yield 2 single chain constructs

Materials: pKC42.3 + INSERT @ 4.2ng/μl (D-825-93)
pKC34.3 " " @ 8.8ng/μl (D-825-74)
pKC35.2 " " @ 11.8ng/μl (D-825-74)
T4 ligase
T4 ligase buffer

Method: Set up 3 ligations with pKC34 and 3 with pKC35 plus 1 vector only ligation.

Vector ≅ 3400 bp. Insert ≅ 850bp. Ratio = 1:4 (vector:insert).

Mixed all components except ligase. Heated 2 minutes @ 65°C. Returned to ice, added ligase, and incubated 1 hour @ room temp.

43 (pKC34.3)	1:0	1:1	1:3	3:1
vector	3.4	3.4	3.4	10.2
insert	—	4	12	4
buffer	1	1	2	2
ligase	1	1	1	1
water	4.6	0.6	1.6	2.8
	10μl	10μl	20μl	20μl

44 (pKC35.2)	1:1	1:3	3:1
vector	3.4	3.4	10.2
insert	3	9	3
buffer	1	2	2
ligase	1	1	1
water	1.6	4.6	3.8
	10μl	20μl	20μl

Conclusion: Ligations set up as described.

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Objective: To transform XL1B cells with pKC43 and pKC44

Materials: pKC43 ligation: D-837-1
pKC44 " "
XL1B cells
B17-1 media

Method: ① Chilled tubes on ice added 100µl cells and ½ of each ligation. Let sit on ice for 30 minutes.

② Heat shocked @ 42°C for 45 seconds. Returned to ice added 1 ml media to each. Let grow @ 37°C with shaking for 1 hour. Plated 10µl and 100µl of each on B17-1 + amp plates. Grown overnight @ 37°C.

Conclusion: Colonies counted

pKC43
1:0 10µl = 0
100µl = 0

1:1 10µl = 74
100µl = TNTC

1:3 10µl = 105* KC
100µl = ~~105~~ TNTC

3:1 10µl = 105
100µl = TNTC

pKC44
1:1 10µl = 103
100µl = TNTC

1:3 10µl = 136*
100µl = TNTC

3:1 10µl = 129
100µl = TNTC

Conclusion: Numbers look good! Grew 10 candidates each of plates marked with *

KC

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Witnessed & Understood by me,	Date	Invented by	Date
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Objective: To induce the Fab constructs containing the D011.10 cell line's TCR (pKC34, 35, 40, and 41) and the SC constructs (pKC43 and 44). pKC18 is a positive control to the β chain.

Materials:

cells all adapted to high-phosphate media
high-phosphate media low-phosphate media
protein extraction buffer (R77) urea

Method: ① Cells grown overnight in high phosphate media @ 30°C with shaking - 5ml each.

Attached: Basic induction protocol.

INDUCTION OF THE *phoA* PROMOTER SYSTEM:

Small scale:

Grow overnight culture in high phos. medium (B5) @ 30°C....5 mls is enoug

② Harvest 1/2 mls into each of two tubes...

Pellet cells (gently)...wash one pellet with high phos. medium, and the other with low phos. medium (B8)...

Repeat once...

Resuspend the appropriate pellet with 1/2 mls high phos. medium, and the oth with 1/2 mls low phos. medium... → only to some constructs: see below

Inoculate the resuspended cells into 50/100mls of the appropriate medium...

Induction is carried out with growth @ 30°C for 4+ hours - overnight.

- **NOTE: Both B5 and B8 already contain *ampicillin* !**

Inductions were carried out for 5 hours & overnight as noted:

5 hr. → 34, 35, 40, 41, 43, 44, 18
5 hr. non-induced → 44
ON → 34, 35, 40, 41, 43, 44, 18
ON non-induced → 44, 18

③ OD 600s were measured:

34-5 (0.04 x 2 = 0.08) →	use all 50ml	= 10 ODs
35-5 (0.07 x 2 = 0.14)	"	"
40-5 (0.15 x 5 = 0.75)	13.3 ml	"
41-5 (0.1 x 5 = 0.5)	20.0 ml	"
43-5 (0.03 x neat = 0.03)	all	"
44-5 (0.1 x 5 = 0.5)	20.0 ml	"
18-5 (0.12 x 5 = 0.6)	16.7 ml	"
44N-5 (0.055 x 10 = 0.55)	18.2 ml	"
34-ON (0.09 x 10 = 0.9)	11.1 ml	"
35-ON (0.21 x 10 = 2.1)	4.8 ml	"
40-ON (0.09 x 10 = 0.9)	11.1 ml	"
41-ON (0.11 x 10 = 1.1)	9.1 ml	"
43-ON (0.05 x 10 = 0.5)	20.0 ml	"
44-ON (0.26 x 10 = 2.6)	3.8 ml	"
18-ON (0.1 x 10 = 1.0)	10.0 ml	"
44N-ON (0.4 x 10 = 4.0)	2.5 ml	"
18N-ON (0.37 x 10 = 3.7)	2.7 ml	"

④ Cells (volumes listed for 10 ODs) were spun down. Pellets resuspended in 1ml protein extraction buffer. Sonicated 2 minutes using cup sonicator. Spun 5 minutes @ maximum speed. Supe. = soluble fraction. Resuspended pellets in 1ml of same buffer + 8M urea. Rocked 1/2 hour @ room temperature and spun 5 minutes @ maximum speed. Supe. = insoluble fraction.

Western Blot + Coomassie Gel of Lysates

Objective: To look @ E coli lysates for the induction of either Fab TCR or single chain TCR.

Materials: lysates prepared on p. 4-5
2x cracking buffer w/ βME blotto
12.5% SDS-PAGE gels membrane

Method: ① Lysates were diluted 1:2 in 2x cracking buffer (10 µl lysate + 10 µl buffer, per lane) Boiled 5 minutes. Run out on 12.5% gels.

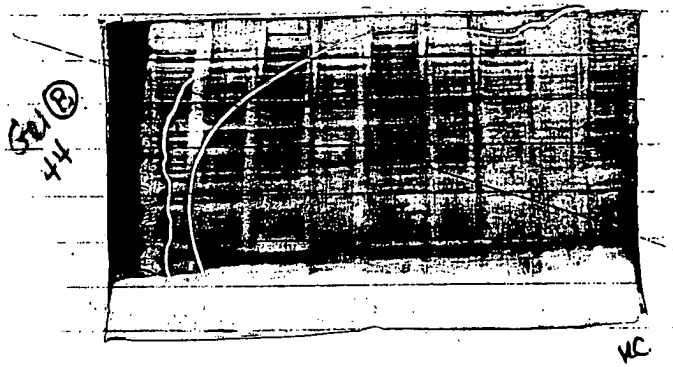
② Transferred to membrane for 15 minutes @ 250-300 mAmps.

③ Blocked 1 hour in blotto diluted 1:10 in 1x PBS. Washed 1x. Probed for 1 hour with α-αβTCR-HRP @ 1:1.5K (26 µl / 39 ml buffer) in blotto diluted 1:50 in 1x PBS. Washed 3x. Exposed to ECL HRP substrate for 1 minute. Exposed film.

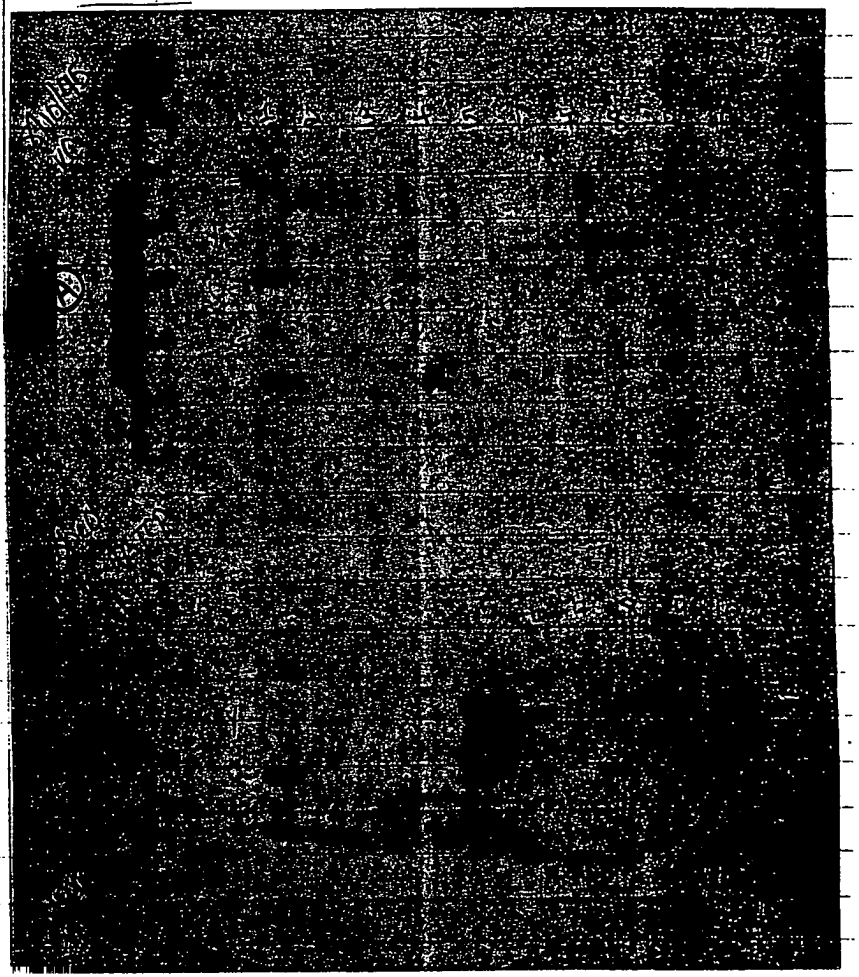
Attached: Coomassie stained gel.

Western Blot + Coomassie Gel of Lysates

Attached: Coomassie blue-stained gel.



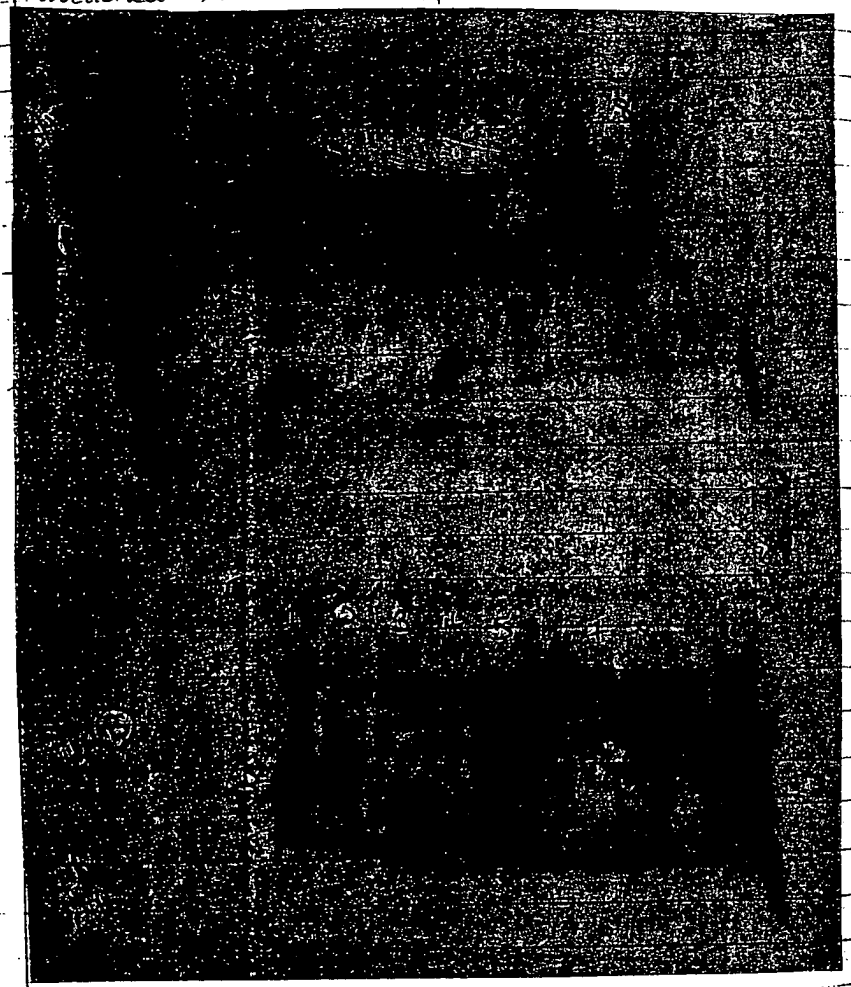
Attached: Blot. 10 minute exposure.



4

Western Blot + Coomassie Gel of Lysates

Attached: Blot. 1 hour exposure.



Gel A

1) mwm
2) 43 I-5 hr. (S)
3) " (I)
4) 43 I-ON (S)
5) " (I)
6) 18 I-5 hr. (S)
7) " (I)
8) 18 I-ON (S)
9) 18 NI-ON (S)
10) 18 NI-ON (I)

Western Blot + Coomassie Gel of Lysates

Gel B

1) mwm
2) 44 I-5 hr. (S)
3) " (I)
4) 44 I-ON (S)
5) " (I)
6) 44 NI-5 hr. (S)
7) " (I)
8) 44 NI-ON (S)
9) " (I)
10) 18 I-ON (I)

I = induced
NI = non-induced
ON = overnight
(S) = soluble
(I) = insoluble

Conclusion: pKC44 has a lot of material in the overnight, insoluble fraction. It is the right size (≈ 50 KD) and may be in the inner membrane, which is where we would like to see it.

The other constructs do not show induction.

KC

Purification of SCTCR using α-Vβ8.2 Antibody Column (SCTCR lot #5)

Objective: Want to try purifying the SCTCR using an α-Vβ8.2 affinity column.

Method: ① started with 22.36 g of fermentor paste from J. Seepschen. Lot = 0-758:91, fermentor #2, 9/13/95.

Decided to resuspend paste @ ≅ 100 OD/ml in buffer. (Buffer = 50mM tris, 150mM NaCl, 0.5% NP40)

≅ 3 liters of culture = 130 grams of cell paste
OD = 30.7/ml
∴ decided to resuspend paste in 150ml

$\frac{3000\,ml}{130\,g} = \frac{x}{22.36\,g}$ $x = 516\,ml \times 30.7\,OD/ml =$ $\frac{15,841.2\,OD}{150ml} = 105.6\,OD/ml$

To the 150ml of buffer, added 3 tablets of "Complete" (protease inhibitor from Boehringer Mannheim). Let dissolve completely.

② Resuspended cell paste in buffer. Passed through French press twice.

③ Let solubilize @ 4°C with shaking/rocking for ≅ 4 1/2 hours.

④ Spun. To soluble fraction, added $CNBr_2$-Vβ8.2 resin (see pages 22-3) and let rock overnight @ 4°C.

Purification of SCTCR

⑤ Split sample into 3-50ml tubes. Spun gently @ 800 rpms for 2 minutes to pull down resin. Saved supe as "flow-thru".

⑥ Pooled resin together in 1 tube and washed 1x with more ~~buffer~~. Transferred resin to column and washed 1x with more buffer.

⑦ Eluted two fractions:
fraction #1 = 1ml of 0.1M glycine + 0.1% NP40
fraction #2 = 2ml " , pH 3.0

Neutralized each fraction with 200µl 2M tris, pH 8.0.

⑧ Split fraction #2 among four microcon-100 concentrators. Spun 30 minutes @ maximum speed @ 4°C. Wash = add 300µl hepes buffer (R50) + 0.1% NP40 and spin as above. Washed 3x.

⑨ Pooled sample from four tubes to two tubes and rinsed membranes. Washed 1x.

⑩ Pooled sample from two tubes to 1 tube. Rinsed membranes and washed 1x. Rinsed membrane checked pH - ok. Final volume = 240µl in hepes buffer + 0.1% NP40.

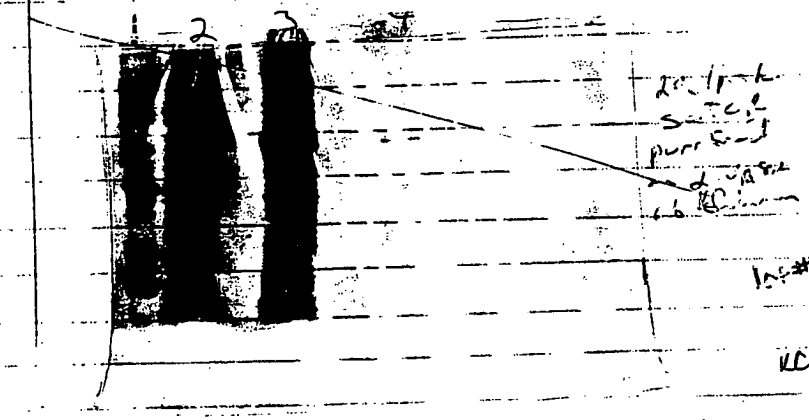
Conclusion: SCTCR prep. (lot #5) prepared.

Coomassie Gel of Purified SCTCR lot#5

Objective: To look @ the purified SCTCR. This lot was purified with the α-Vβ8.2 Ab.

Method: KC Same as on page 74 but did not run a western blot.

Attached: Coomassie stained gel.



1 = mwm
2 = lysate
3 = flow-thru
4 = purified SCTCR (18 µl / 240 µl total)

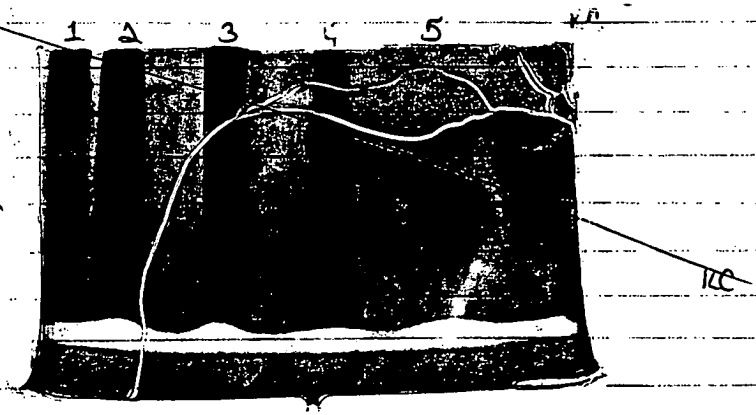
Conclusion: Can see purified SCTCR band!

Western Blot + Coomassie Gel of SCTCR (lot #6)

Objective: To look @ the purified SCTCR. This lot was purified with both the α-Vβ8.2 and α-αβTCR Ab columns combined.

Method: Same as on page 74.

Attached: Coomassie stained gel.



1 = mwm
2 = lysate
3 = flow-thru
4 = wash*1
5 = purified SCTCR

Conclusion: Purified SCTCR looks good!